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REVIEWS

Review

Experimental Models of Impaired Hypoglycaemia-Associated Counter-Regulation

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Impaired awareness of hypoglycaemia (IAH) affects around a quarter of patients with diabetes who receive insulin treatment. This condition is characterised by a progressive reduction in symptomatic and behavioural responses to hypoglycaemia, increasing risk of deeper drops in blood glucose, unconsciousness, and collapse. Thus, patients with IAH experience severe hypoglycaemic episodes more frequently, resulting in significant morbidity and mortality. IAH is thought to develop as a consequence of whole-body adaptations to repeated insulin-induced hypoglycaemia (RH), with widespread deficits in the hypoglycaemia counter-regulatory response (CRR). Despite this important insight, the precise pathophysiology by which RH leads to an attenuated CRR is unknown. Studies into the underlying mechanisms of IAH have employed a variety of protocols in humans and experimental species. The use of animal models has many investigational benefits, including the unprecedented increase in the availability of transgenic strains. However, modelling impaired hypoglycaemia-associated counter-regulation remains challenging and appropriate interpretation of findings across species and protocols even more so. Here, we review the experimental modelling of IAH and impaired hypoglycaemia-associated counter-regulation, with a focus on understanding species-specific variation in glucose homeostasis. This review will aid investigators in interpreting outputs from different studies in IAH and aid progress in the field.

Highlights

Impaired awareness of hypoglycaemia (IAH) is a common and poorly understood complication of insulin-treated diabetes and evolves following repeated episodes of hypoglycaemia.

An improved mechanistic understanding of IAH is required and necessitates a robust disease model with which to interrogate changes within the glucose-regulatory network.

A large variety of experimental models have been outlined, leading to a lack of consensus among investigators in this field. Appreciating the influence of experimental parameters and physiological differences on findings can aid progress in the field.

Hypoglycaemia Counter-Regulation and IAH

For individuals with diabetes, **hypoglycaemia** (see Glossary) is a relatively common adverse effect of insulin therapy, occurring as frequently as twice per week in those with type 1 diabetes [1,2]. Hypoglycaemia leads to both acute and chronic multisystemic effects, causing significant morbidity, mortality, and distress to patients [1]. The increased propensity for hypoglycaemia occurs in patients with type 1 diabetes and long-duration type 2 diabetes in a setting of markedly altered glucose homeostasis. First, pancreatic β cell destruction or decline necessitates insulin administration which, despite advances, is normally delivered as a subcutaneous (SC) depot at supraphysiological doses. This leads to relative systemic hyperinsulinaemia, increasing peripheral uptake of glucose and, therefore, the risk of hypoglycaemia. Crucially, exogenous insulin is not then supressed, as would normally occur when the blood glucose falls below 4.4 mmol/l [3]. Second, in patients with type 1 diabetes, α cell dysfunction develops, impairing normal glucagon release. This deficit affects most patients within 5 years of disease diagnosis [4]. This places a greater reliance on the sympathoadrenal system and adrenaline release for hypoglycaemia-induced **CRR** (Box 1) [5]. Subsequently, the loss of glucagon and sympathoadrenal system responses is closely correlated with the loss of symptom responses, leading to **IAH**.

IAH is highly prevalent, affecting around 25% of patients undergoing insulin treatment for diabetes [8]. Individuals with IAH have a sixfold increased risk of severe hypoglycaemia, leading to acute and chronic health sequelae [8]. However, identifying patients with IAH can be tricky, as

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Box 1. The Normal Response to Falling Blood Glucose Levels

In healthy individuals, a fall in blood glucose initiates a CRR. This takes the form of hormone release or inhibition, as well as symptom awareness, prompting appropriate behaviours (see Figure 1 in main text). If the blood glucose falls below 4.5 mmol/l, endogenous insulin secretion is inhibited. This is mediated not only through peripheral mechanisms, but through supplementary control from the brain [6]. This reduces glucose uptake by nonneuronal tissues and for most individuals is sufficient to maintain their blood glucose in the normal range. However if the blood glucose falls below 3.8 mmol/l, the glucose-sensing network in the central nervous system initiates a hormonal CRR [7]. This includes glucagon release from pancreatic α cells and adrenaline release from the adrenal gland chromaffin cells [7]. This is soon followed by hypoglycaemia autonomic symptoms (see Figure 1B in main text), which are a vital response for individuals with diabetes. Autonomic symptoms primarily arise from sympathoadrenal system activity and develop at a glucose level of ~3.2 mmol/l [3]. They lead to awareness and a prompt behavioural response from the individual (carbohydrate ingestion or to seek assistance) before cognitive dysfunction and neuroglycopenia occurs at ~2.7 mmol/l [3]. Thus, individuals who retain symptom awareness of hypoglycaemia are able to take action before cerebral dysfunction occurs.

Glossary

Counter-regulatory response (CRR): the stepwise autonomic and neuroendocrine mechanisms that act during hypoglycaemia to restore normal blood glucose (Box 1).
Hypoglycaemia: blood glucose of less than 3.9 mmol/l with (documented symptomatic hypoglycaemia) or without (asymptomatic hypoglycaemia) symptoms.
Impaired awareness of hypoglycaemia (IAH): diminished ability to perceive the onset of hypoglycaemia.

hypoglycaemia symptoms are subjective and can be present or absent to varying degrees [9]. Hypoglycaemia symptoms can be categorised into three groups: autonomic, neuroglycopaenic, and malaise [10]. Autonomic symptoms (Figure 1B) are a result of increased sympathoadrenal activity (endocrine adrenaline release and sympathetic neural activity). They occur at blood glucose levels of ~3.2 mmol/l and serve to 'raise the alarm', prompting a behavioural response (food ingestion). In their absence, blood glucose levels may decrease further, precipitating neuroglycopaenia. Neuroglycopaenic symptoms (Figure 1B) occur due to brain depletion of glucose and become apparent at blood glucose levels of ~2.7 mmol/l. In IAH, autonomic symptom impairment can be explained by the seminal discovery that antecedent hypoglycaemia leads to a reduced adrenaline response, as well as symptoms, to subsequent hypoglycaemia [11]. Repeated hypoglycaemia (RH) lowers the glycaemic threshold at which both autonomic and neuroglycopaenic symptoms are perceived. Ultimately, neuroglycopaenia may occur first, preventing the recognition of autonomic symptoms [12]. Therefore, RH is likely to be an important driver of IAH in diabetes, leading to altered magnitude and threshold of the sympathoadrenal response.

Unfortunately, traditional treatment options for IAH have limited efficacy and are often to the detriment of controlling the underlying diabetes. Achieving an effective treatment for IAH, it can be argued, will first require a greater understanding of IAH at a mechanistic level. Establishing the specific effects of RH on the whole-body glucose-regulatory network requires robust models.

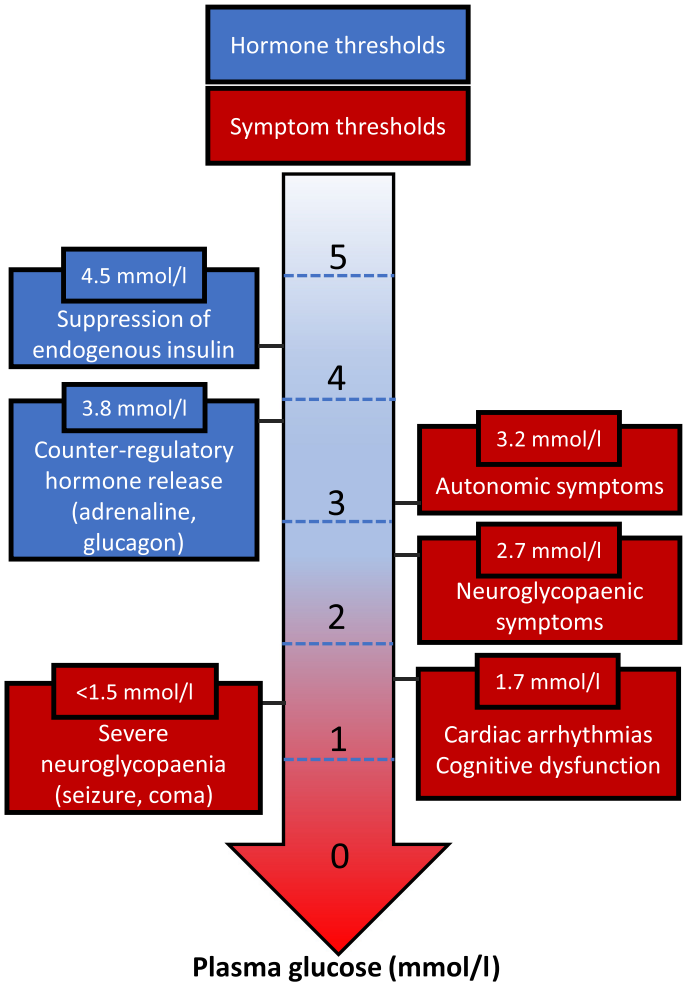
Experimental Approaches to Investigate IAH

Following the seminal work that identified that two prior hypoglycaemic episodes lead to diminished sympathoadrenal and symptom responses to subsequent hypoglycaemia, human (Box 2) and animal studies have adapted this experimental design in a number of ways to more fully investigate IAH [13–16].

Measuring IAH and the Impaired Hypoglycaemia CRR

Central to the investigation of IAH has been the search for a robust measure for impaired hypoglycaemia CRR and/or IAH. The use of symptom scores such as the Edinburgh Hypoglycaemia Scale allows a degree of quantification of hypoglycaemia awareness [8]. Symptom scores are limited in that they are culturally specific and vary based on subjective interpretation and prior experiences. In addition, symptom responses are affected by experimental conditions, particularly when measured in a highly controlled laboratory environment and following overnight fasting. Also, it can be argued that symptom scores do not fully encompass the magnitude of impairment of the CRR with RH. Therefore, biological markers of IAH and impaired CRR may have more utility, allowing greater comparison between studies.

(A)



(B)

Autonomic	Neuroglycopaenia	Malaise
Sweating Palpitations Shaking/tremor Hunger	Confusion Drowsiness Speech difficulty Incoordination Odd behaviour	Headache Nausea

Trends in Endocrinology & Metabolism

(See figure legend at the bottom of the next page.)

Box 2. Investigating IAH in Humans

In humans (both healthy individuals and patients with diabetes), RH-related changes are always studied with the aid of hypoglycaemic–hyperinsulinaemic clamps. This technique ensures that blood glucose levels are maintained at a desired threshold and, importantly, ensures safety. The aim in human studies of IAH is to expose subjects to either a SH or multiple antecedent episodes of hypoglycaemia on the first day, followed by a subsequent SH episode on the following day. Counter-regulatory hormones (adrenaline, glucagon, and corticosterone) and hypoglycaemia symptom scores are measured following both the antecedent and the subsequent hypoglycaemia to allow IAH and the impaired CRR to be characterised [13–16].

In humans, neuroimaging modalities have been employed to investigate changes in brain activity, metabolism, and blood flow following SH and RH, providing insights into hypoglycaemia-responsive brain regions [17]. Interestingly, other measures of nervous system changes have also been studied, identifying decreased baroreflex sensitivity and muscle sympathetic nerve activity following two episodes of antecedent hypoglycaemia [16]. These data support the hypothesis that the effects of antecedent RH are on multiple arms of the nervous system. This generalised effect of RH on the autonomic and neuroendocrine systems is also demonstrated by the finding that comparable impairments of hormonal and symptom responses are seen in individuals both with and without diabetes [11,15,18]. In addition, this hints that the changes that follow RH are not specific to the pathophysiology of diabetes, but rather a reflection of whole-body adaptation to the repeated stress of hypoglycaemia.

A major limitation of studies in diabetic subjects and in patients with IAH is that it is not known whether unidentified hypoglycaemia episodes have occurred prior to the beginning of the study. As a result, glucose-regulatory network changes may already be present to a varying degree prior to the study, affecting findings. Despite these limitations, human studies have provided important insights into the organisation of the glucose-regulatory network and the hypoglycaemia CRR. However, to enable more detailed mechanistic and cellular understanding of the pathophysiology of IAH, animal models are required.

At a mechanistic level, there is consensus in the field that impaired sympathoadrenal responses and loss of glucagon secretion largely account for the loss of symptomatic awareness that characterises IAH [19]. The sympathoadrenal network comprises sympathoneural and adrenomedullary components, which are both impaired with RH. With hypoglycaemia, both arms of the sympathoadrenal system are activated, although it has been suggested that the sympathoneural system is capable of generating autonomic symptoms of hypoglycaemia, even in the absence of a functioning adrenomedullary system [20]. Hypoglycaemia-related symptoms and counter-regulation are mediated through the effects of catecholamines, particularly adrenaline and noradrenaline. These catecholamines normally increase in parallel following hypoglycaemia, such that plasma levels of adrenaline and noradrenaline are highly correlated [20]. However, in rats, insulin-induced hypoglycaemia may preferentially stimulate adrenomedullary cells producing adrenaline compared with those producing noradrenaline [21]. Similarly, both central and systemic glucoprivation with 2-deoxyglucose (2DG) treatment in rats increases adrenaline secretion preferentially over noradrenaline [22–24]. This detail may be species related or due to difficulties in detecting differences in plasma noradrenaline [25]. Therefore, adrenaline in concert with noradrenaline leads to potent glucose-raising effects, which, in the setting of deficient glucagon release, are vital for patients with type 1 diabetes.

Quantification of the sympathoneural response is possible by measuring noradrenaline spill over from synaptic release into the circulation. However, this technique may underestimate sympathetic activation, as the majority of the released noradrenaline is dissipated locally by reuptake

Figure 1. Hormone and Behavioural Responses to Falling Blood Glucose. (A) Stepwise hypoglycaemia and thresholds for each counter-regulatory response (CRR) in humans. (B) Autonomic, neuroglycopaenic (shortage of glucose in the brain due to hypoglycaemia), and malaise symptoms. Normally, autonomic symptoms are initiated, thereby raising awareness of hypoglycaemia. However, in patients with impaired awareness of hypoglycaemia (IAH), thresholds are dangerously altered, with neuroglycopaenic symptoms and injury occurring before autonomic symptoms (which are often absent altogether) [3,10,76,96].

mechanisms and local metabolism and does not enter the circulation [26]. Thus, sympathetic activation sufficient to produce biological effects may not be reflected by a concomitant rise in plasma noradrenaline [27]. This has been demonstrated for hypoglycaemia thresholds, which must be markedly reduced (<2 mmol/l) before increases in pancreatic noradrenaline are detected [27]. Thus, the relative ease and accuracy of measuring plasma adrenaline, compared with noradrenaline, makes it the best biomarker for RH-related impaired glucose-counter regulation and IAH [28].

Glucagon, which is the other major counter-regulatory hormone, also is measured consistently in studies investigating IAH. RH leads to attenuation of glucagon release in a subsequent episode of hypoglycaemia in most nondiabetic human studies [17,18,29,30]. However, the relevance of this in the context of IAH is diminished, as glucagon release ceases within 5 years of diagnosis in most patients with type 1 diabetes. This glucagon deficit appears to occur through a mechanism distinct from the RH effect on catecholamine responses [31]. Therefore, patients with type 1 diabetes may develop attenuated glucagon release before IAH develops. This reiterates the importance of the sympathoadrenal response in people with diabetes who have IAH [32].

Cortisol (corticosterone in rodents), the major 'stress' hormone, also increases following hypoglycaemia [33]. In general, in humans, antecedent insulin-induced hypoglycaemia attenuates cortisol release in a subsequent episode of hypoglycaemia [14,17,32,34,35]. However, this is not a universal finding and other investigators have reported no change in cortisol release with RH [30]. Hypothalamopituitary–adrenocortical (HPA) axis activation typically occurs as the blood glucose falls to <3.7 mmol/l; however, the effects of cortisol on blood glucose are delayed. Using a pancreatic-adrenocortical-pituitary clamp to maintain basal cortisol levels, De Feo *et al.* demonstrated that a lack of cortisol rise resulted in lower rates of glucose production and higher rates of glucose utilisation only after 6 h in nondiabetic humans (glucagon, insulin, and growth hormone were infused to maintain similar plasma concentrations in the two groups) [36]. In addition, cortisol-deficient patients only begin to display reductions in plasma glucose (compared with controls) 2.5 h after insulin-induced hypoglycaemia [37]. These findings suggest that the counter-regulatory effects of cortisol are more relevant during prolonged hypoglycaemia, which patients often encounter while asleep [38]. Equally important is that HPA axis activation does not initiate hypoglycaemia-symptom awareness. Therefore, it can be argued that cortisol/corticosterone measurement in studies relating to IAH is also of minor relevance.

Animal Models of Loss of Hypoglycaemia-Associated Counter-Regulation

Studying the condition of IAH using animal models provides several advantages to investigators. Using experimental tools, RH-related changes at a tissue and cellular level can be observed. This is particularly true for studies relating to the central nervous system, which may hold the key to understanding IAH. Animal studies also allow glucose counter-regulation to be measured at a wider range of glucose levels and under more controlled experimental conditions. Last, therapeutic discovery requires an effective animal model prior to consideration for human intervention. Among the animal species studied, rats are most commonly encountered in IAH research, with fewer mouse studies published.

In rodent studies, investigators have modified human experimental protocols of RH to investigate CRR changes. These protocols, which involve between one and 12 antecedent insulin-induced hypoglycaemia episodes, demonstrate that, in rodents, impairments of the CRR can be achieved analogous to the human phenomenon [25,39–57]. This is particularly true for plasma adrenaline and, in general, for plasma corticosterone, although a small number of studies have reported no effect of RH on plasma corticosterone release [52,53]. Similarly, there are inconsistencies

in eliciting impairment of the glucagon response, an observation that is consistently reported in both human and mouse studies [13,14,17,29,30,35,58–62]. As impairment of the sympathoadrenal response and adrenaline release is considered the best marker for IAH and impaired counter-regulation, this suggests that rodent models can provide a good representation of the human condition. One notable problem is that identifying and recording awareness is much more difficult than in human studies of IAH. Otlivanchik *et al.* have used conditioned place preference (CPP) behaviour, and its loss with single hypoglycaemia (SH), as a surrogate measure of hypoglycaemia awareness. In their model CPP is first induced by giving rats a reward to induce place preference. The CPP is blunted with the negative perception of SH, reversing the reward conditioning. However, if the rats first undergo RH, they are no longer 'aware' of the negative valency of a subsequent hypoglycaemic episode and the CPP is preserved [63]. As our understanding of animal behaviours improves, more direct hypoglycaemia symptom measures may be identified, allowing more accurate modelling of the clinical condition.

In rats, not all protocols utilise insulin to study RH-related changes. In some studies, insulin is substituted or occasionally combined with repeated injections of 2DG, a strong glucoprivic agent [57,64,65]. Consistent with human studies, rodents exposed to repeat insulin-induced hypoglycaemia and then perfused with 2DG intracranially, demonstrate significant suppression of the CRR [66]. However, 2DG causes an increase in peripheral glucose and has numerous intracellular effects independent of inhibiting glucose metabolism [67].

From rat studies, we can gather that, despite differences in protocols, many groups have achieved attenuation of the adrenaline response following RH, the key marker of successful modelling of the disease [25,39–41,43,44,46,47,50–52,54,68]. A recent protocol outlined by the McCrimmon laboratory to study IAH and RH-related counter-regulatory impairment in rats, used intraperitoneal (IP) insulin (0.75–1 units/kg, Novorapid) or volume-matched IP saline injections administered three times weekly for 4 weeks. The chronicity of the protocol, combined with its validation in a type 1 diabetes animal model, make it more relatable to the human condition. Crucially, use of this protocol significantly attenuated the adrenaline response to subsequent hypoglycaemia, fulfilling the criteria for successful modelling of the human condition [69].

For many groups, translation from rat models to the mouse has proved to be more difficult. However, the development of a mouse model is of interest, as transgenic strains and recombinant techniques enable unprecedented appreciation of pathways, including those implicated in glucose homeostasis.

Poplawski *et al.* utilised a RH protocol involving daily IP insulin injections for 4 days before comparing responses on the fifth day between the RH and a SH group. This protocol produced attenuated glucagon and corticosterone release, but no impairment of the adrenaline response to subsequent hypoglycaemia was noted [62]. In this study, hormones were measured from truncal sampling 4 h after insulin injection, which is likely to have identified more delayed hypoglycaemia-related hormone effects [62].

Experiments by a different group, in chronically cannulated wild-type and transgenic mice, have examined hypoglycaemia CRR hormone release following a single prior hypoglycaemic episode. In these experiments, mice received either a euglycemic or a hypoglycaemic clamp on day 1 and CRR hormone responses were measured following a subsequent hypoglycaemic clamp on day 2. Antecedent hypoglycaemia significantly lowered glucagon but not adrenaline in both experiments [61,70]. The authors in these studies have argued that all components of the CRR

need not be significantly changed in a mouse model of IAH and impaired CRR [61,70]. To date, only Ma *et al.* have been able to demonstrate diminished 24-h urinary adrenaline levels following a 4-day protocol of repeated IP insulin injection [65]. Truncal blood (plasma) adrenaline was closely correlated with urinary adrenaline. However, this was not significantly attenuated with RH, but was reduced with antecedent 2DG. This discrepancy raises the question of whether the acute CRR to hypoglycaemia was truly impaired [65]. Therefore, a robust murine model is yet to be described, although the Luckman laboratory has now replicated the 4-week protocol in mice previously reported by the McCrimmon laboratory in rats [69].

Animal studies, and particularly those in rats, have been instrumental in investigations of the mechanisms underlying IAH. However, they are not without their own disadvantages. Species- and strain-specific physiological differences are underappreciated. In addition, animal studies commonly lead to loss of biological variability, a result of using inbred strains and young animals (often littermates) and restriction to a single sex. Other influential factors include the need to control for handling and environmental stressors – particularly relevant when measuring responses of the sympathoadrenal system [71].

Experimental Protocols and Parameters in IAH Studies

Important differences exist in how IAH and RH-related counter-regulatory impairment is achieved between human and rodent studies. In humans, the general protocol for achieving impaired CRR involves exposing subjects to one or two antecedent episodes of hypoglycaemia on the previous day, followed by a subsequent episode of hypoglycaemia [13,14,18,29,30,32,35,58–60,72]. Following an overnight fast, reductions in blood glucose to between 2.2 and 3.3 mmol/l, for about 1–2 h, are achieved utilising a hyperinsulinaemic–hypoglycaemic intravenous (IV) clamp. Venous blood sampling allows the measurement of counter-regulatory hormones and symptom scores are recorded [13,14,18,29,30,32,35,58–60,72]. However, direct translation of the above experimental protocol to rodents has been demonstrated by only two independent groups, signifying the challenges faced by investigators [46,49]. These difficulties may be explained by differences in intrinsic inter- and intraspecies variation in glucose and CRR physiology, an area of research that requires much greater attention (Box 3).

Box 3. Interspecies and Interstrain Differences in Glucose Homeostasis and CRR

The BMR determines the glucose and energy requirements for an organism to maintain body function. In relative terms, the mouse has a BMR that is around 7.5 times greater than that of a human [73]. This is reflected by the finding that mouse blood glucose levels are substantially higher than in humans and that the relative basal glucose turnover rate is 10–15 times greater [74]. This increased glucose requirement is met, in part, by enhanced gluconeogenesis. Corresponding with the enhanced BMR and glucose turnover, mice have higher endogenous insulin production than rats and humans [74]. Mice and humans display more similarities with respect to hormone thresholds. In particular, thresholds for glucagon secretion in response to insulin-induced hypoglycaemia are comparable [75]. However, in mice the glucose threshold for an increase in plasma adrenaline and corticosterone is around 4.4 mmo/l [61]. This is higher than the 3.3–3.9-mmol/l glucose threshold reported in nondiabetic humans [76]. Precise hormone release thresholds have not been identified in rats. However, it is evident that rats demonstrate significant increases in adrenaline, glucagon, and corticosterone when hypoglycaemia ~3.2 mmol/l is achieved, paralleling the nondiabetic human condition [25,39–57].

The assessment of strain-dependent metabolic variance has been performed more extensively in mice than in rats. In the latter, background strain effects have been more frequently studied in the context of experimental obesity. Here, even in the commonly used Wistar and Sprague–Dawley rats, diet-induced obesity and insulin resistance susceptibility vary considerably [77]. In mice, baseline glucose production and turnover and endogenous insulin secretion have been shown to differ between commonly used mouse strains (ICR, FVB/N, 129X1/Sv, and C57BL/6) [78]. Following insulin-induced hypoglycaemia, adrenaline release was increased in C57BL/6, FVB/N, and DBA/2 mice but was below the limits of detection in 129X1/Sv mice [79]. Glucagon release to hypoglycaemia was similarly blunted in 129X1/Sv mice, while corticosterone release was transiently elevated in DBA/2 mice but did not increase in C57BL/6, 129X1/Sv, and FVB/N mice [79]. This nicely highlights that background strain, as well as species, can significantly alter hypoglycaemia counter-regulation, which will directly impact CRR hormone levels following RH.

A summary of pertinent studies in humans, rats and mice illustrating the diversity of protocols is shown in Table 1. We now consider how differences in RH protocol parameters, along with species-specific differences in physiology, influence CRR output measures.

Depth and Number of Antecedent Hypoglycaemia Episodes

The use of antecedent hypoglycaemia depths of <2.0 mmol/l (profound hypoglycaemia) is also encountered in the literature, although this degree of hypoglycaemia, which is often associated with multiple seizures, is of debatable relevance to the human condition and may lead to irreversible changes [25,39,41,46,49,52,53]. In humans, the antecedent hypoglycaemia depth has been shown to influence both the magnitude and the pattern of CRR. Davis *et al.* reported segregation of patterns of hormone attenuation depending on the depth of antecedent hypoglycaemia in humans. Specifically, hypoglycaemia of 3.9 mmol/l during antecedent episodes led to reduced plasma adrenaline and glucagon responses to subsequent hypoglycaemia, whereas with blood glucose levels of <3.3 mmol/l a more significant reduction in glucagon, adrenaline, noradrenaline, and growth hormone responses occurred following subsequent hypoglycaemia [80]. Interestingly, there was no additional impairment in the counter-regulatory hormone response when the antecedent hypoglycaemia depth was reduced further, from 3.3 to 2.9 mmol/l. This suggests that there is a hierarchy of counter-regulatory hormone impairment, which depends on the depth of the antecedent hypoglycaemia. Whether this hierarchy continues with antecedent hypoglycaemia depths <2.9 mmol/l is unknown [80].

In humans, the duration of antecedent hypoglycaemic episodes also affects the pattern of counter-regulatory impairment. Of note is that short (5 min), intermediate (30 min), and prolonged (90 min) antecedent hypoglycaemic episodes all produced impairments comparable with counter-regulatory hormones [81]. However, autonomic symptoms were blunted significantly only by intermediate- or prolonged-duration hypoglycaemia [81]. If these observations translate to animals, hypoglycaemia durations of between 30 and 90 min should also attenuate CRR in animals. Most successful animal studies employ durations of between 60 and 180 min of hypoglycaemia in their protocols [25,39–57].

There is evidence to support greater impairment of hypoglycaemia counter-regulation when the number of antecedent hypoglycaemic episodes is increased in humans [34,80,82]. Similarly, in

Table 1. Summary of Experimental Protocols for RH-Related Impaired CRR in Humans, Rats, and Mice[a]

Species	Route	Insulin dose	Fasting	Measurement method	Duration of hypoglycaemia	Depth of hypoglycaemia	Number of antecedent hypoglycaemia events	Refs
Human studies	IV	1–2 mU/kg/min (all studies)	Nonfasted (three studies) Fasted (all other studies)	Venous systemic	40 min to 2 h (majority of studies) 3 h (one study)	2.2–3.3 mmo/l (all studies)	1/day for 1 day 2/day for 1 days (majority of studies) 1/day for 4 days	[13,14,18,29,30,32,35,58–60,72]
Rat studies	IV or IP (majority of studies) SC, ICV[b],or SC 2DG	0.75–10 units/kg 1–3 units/kg (majority of studies) 2DG (200 mg/kg)	Overnight 4 h Nonfasted	Central venous (majority of studies) Tail Trunk	1–3 h duration	2–3.2 mmol/l (majority of studies) 1.4–3.2 mmol/l	1/day for 1 day 1/day for 2 days 1/day for 3 or 4 days 2/day for 1 day Three times per week for 4 weeks	[25,39–57]
Mouse studies	IP and IV	2.5 units/kg 20 mU/kg/min	3–6 h	Central venous Tail	2–3 h duration	2.2–3.8 mmol/l	1/day for 4 days 1/day for 1 day	[61,62,65,70]

[a]Human studies include those undertaken in healthy volunteers and patients with diabetes. Animal studies include those undertaken in transgenic strains and diabetes animal models.
[b]Abbreviation: ICV, intracerebroventricular.

rodents, RH leads to an increase in the depth of hypoglycaemia with successive episodes, risking nonrecovery in the animals. This is reflected in adaptations of protocols such that, as the protocol progresses, the dose of insulin is reduced [69,83]. In our hands, in a 4-week RH protocol where mice receive three insulin injections per week for 4 weeks, there is a greater duration and depth of hypoglycaemia with successive antecedent hypoglycaemic episodes. In rats, after 3 weeks of this RH protocol, the dose of insulin is decreased by 25% to prevent the development of severe hypoglycaemia requiring rescue [69]. Interestingly, the effect of two antecedent hypoglycaemic episodes on the same day, compared with one, does not seem to produce a greater attenuation in adrenaline release on subsequent hypoglycaemia in rats [84]. Therefore, if IAH represents a form of neuronal adaptation such as habituation, an effect may be encountered only with a greater number of hypoglycaemia episodes and by allowing more time between episodes for cellular modification.

Induction of Hypoglycaemia

Repeated insulin-induced hypoglycaemia is accepted as the best model for studying IAH. Although protocols with 2DG-induced glucoprivation exist, this approach does not fully replicate the human condition, where iatrogenic insulin (combined with inherent diabetes-related counter-regulatory deficits) causes hypoglycaemia. In human studies, insulin is administered via IV infusion at a dosage of 1–2 mU/kg/min, without exception. However, in animal studies that utilise IV insulin infusion, dosages of 20–50 mU/kg/min are utilised commonly [41,42,44,45,48,51]. Direct translation of insulin dosage to animals is understandably not possible. However, extreme hyperinsulinaemia, out of proportion to levels witnessed in the human condition, may fundamentally alter physiological responses. Insulin itself can affect the CRR to hypoglycaemia, including modulation of the sympathoadrenal response to hypoglycaemia by shifting glucose-sensing neuron thresholds [85–87]. Also, it has been suggested that hyperinsulinaemia alters the glucagon response to hypoglycaemia in non-diabetic and diabetic humans [3,88,89]. Therefore, to minimise the effect of hyperinsulinaemia on the interpretation of the CRR, it is advisable to utilise the lowest insulin dosage that elicits consistent hypoglycaemia in the chosen protocol.

Animal studies in the field of IAH also employ routes of insulin administration other than IV, including the use of IP and SC injections. The IV route provides advantages in ensuring that precise target hypoglycaemia levels and durations are reached, while providing vascular access for blood sampling and replacement. The IV route is limited in that it can result in stress to animals from surgical implantation. In addition, the procedure makes it complex to prepare large cohorts for investigations. As a result, IP and SC routes are commonly selected due to the ease of administration without the need for prior surgery. There remains a lack of consensus over whether IP or SC insulin produces the more consistent duration and intensity of hypoglycaemia. Flanagan *et al.* report that the IP route was more consistent in their experience; however, other investigators have used SC insulin with good effect [39,40,90]. The IP route increases the risk of injection into the bowel, leading to potential complications and variable delivery of insulin. In the Luckman laboratory, we have found that the SC route can reliably lower blood glucose in a reproducible manner in mice on the C57BL/6 background, leading to a subsequent diminished adrenaline response (S.M. Luckman *et al.*, unpublished). In addition, the SC route replicates how patients administer insulin therapy.

It is known that the administration route directly influences pharmacokinetics. In humans, IV infusion causes significantly higher serum insulin levels than the same dose through SC administration, and SC insulin takes up to four times longer to return to baseline levels [91]. Therefore, the route of administration can alter the bioavailability, affecting the depth

and duration of hypoglycaemia, resulting in variation in counter-regulatory hormone release and impairments following RH [91].

Overnight fasting is the established procedure prior to insulin-induced hypoglycaemia in human studies. In animal studies, investigators use a variety of approaches, including prior overnight fasting [41,45,46,48], fasting for 1–6 h [43,61,62], and even no fasting [25,39,40,47,49,54]. Fasting prior to insulin-induced hypoglycaemia increases tissue sensitivity to insulin and lowers the baseline serum glucose, reducing the total insulin dose requirements to induce hypoglycaemia [87]. In mice, insulin sensitivity increases when the duration of fasting is increased [88]. This may improve the ability to detect hypoglycaemia-specific changes to counter-regulation by limiting the effects of hyperinsulinaemia. However, care must be taken over the duration of fasting. Rodents consume most of their energy requirements during the dark phase of the day–night cycle, which means that overnight fasting is an intense metabolic stressor. Following overnight fasting, rodents deplete liver glycogen stores rapidly, requiring increased *de novo* glucose production [88]. This, in combination with increased insulin-stimulated glucose transport, leads to altered counter-regulatory hormone levels with overnight fasting [88]. In mice, fasting (overnight/>16 h) significantly increases corticosterone and autonomic drive, enhancing glucose production to maintain the basal metabolic rate (BMR) [89,90]. Fasting will, therefore, affect baseline adrenaline and corticosterone, although this is unlikely to mask hypoglycaemia-related increases in these hormones. After 16–18 h of fasting, mice move into a catabolic state, losing significantly more total body, lean, and fat masses than with 5-h fasting [88]. This differs to humans, where prolonged fasting impairs insulin-stimulated glucose utilisation [88]. Thus, in the investigation of responses to RH, overnight fasting may prove to be an excessive stress stimulus and potentially confound findings.

In our laboratory, we routinely fast for 3 h prior to inducing hypoglycaemia, which we have found to reduce baseline glucose and variability between mice.

Considering the diversity of protocols and CRR measures, the focus should remain on ensuring that experimental methods demonstrate a consistent impairment of the sympathoadrenal response following RH. This is the central feature, which we argue demonstrates a robust disease model. However, the model must control for other repeated stressors encountered, to ensure that the impaired sympathoadrenal response is specific to RH. Differences in model parameters, such as route, fasting duration, hypoglycaemia duration, and even the number of antecedent episodes, may be less relevant.

Although IAH and impaired CRR can be modelled in nondiabetic rodents and humans, the findings should ultimately be replicated in the setting of diabetes to ensure translational relevance. This poses additional challenges, which are explored in Box 4. In IAH research, only a few groups have utilised diabetic animal models for their investigations [55,56,92,93]. Here, as in nondiabetic IAH studies, a greater uniformity of approach may enable better comparisons between studies.

Concluding Remarks and Future Perspectives

In this review, we have outlined the challenges faced by investigators in creating a model to study IAH and impaired CRR. Differences in experimental protocols and species and strain variations between studies lead to difficulty in interpreting findings. Here, we have examined the effect of these differences on the CRR and sympathoadrenal response. We argue that the demonstration of the core feature of IAH – namely, impaired plasma adrenaline release – signifies a robust IAH disease model. However, with improved understanding of animal behaviours, it may be possible to identify a direct measure for hypoglycaemia symptoms as well. Despite these challenges,

Outstanding Questions

What is the cellular mechanism by which repeated exposure to hypoglycaemia leads to IAH development?

Where in the glucose-regulatory network does the pathophysiological process occur?

Does glucagon dysfunction in type 1 diabetes alter the glucose-regulatory network and the sympathoadrenal response?

Can we develop a robust measure to assess hypoglycaemia symptoms in animals, thereby allowing a direct measure of hypoglycaemia awareness in the setting of RH?

Box 4. Animal Models of Type 1 Diabetes in IAH Research

A number of diabetic animal models have been identified to allow the study of type 1 diabetes [93]. Here, the main goal is to demonstrate β cell destruction or failure. This can be achieved through a variety of methods, including chemical destruction of β cells, spontaneous autoimmunity through breeding, and genetically induced mechanisms [94]. Streptozotocin-induced diabetes is the most common, chemically induced type 1 diabetes model. It is a simple and cheap method, achieving a high percentage of destruction of β cells [94]. Its major limitation is that the magnitude of β cell destruction can be variable, affecting the severity of insulin deficiency and the resultant diabetes [94]. Unfortunately, insulin maintenance treatments are often not instituted in streptozotocin-induced diabetes experiments, which means that animals are both hypercatabolic and hyperosmotic, leading to weight loss, dehydration, and ketosis– an omission that has direct effects on counter-regulation [56,68]. Specifically, in untreated diabetic rats, glucagon, adrenaline, and corticosterone responses and the subsequent glucose production in hypoglycaemia are reduced compared with normal rats [56,68]. In IAH research, only a few groups have utilised diabetic animal models for their investigation; specifically, streptozotocin-induced diabetes [56,92,93,95]. Although these studies have increased knowledge in the field, they have not increased diabetes-specific understanding of IAH. Despite these challenges and a need for optimisation in approach, replication of nondiabetic model findings in a robust diabetic animal model remains a priority for studies in IAH. We recommend that insulin replacement is used in all animal models of type 1 diabetes to reflect the human condition more accurately and to minimise confounds related to marked insulin deficiency.

several independent groups have demonstrated effective animal models of IAH and impaired CRR [39,43,46,51,56,65,69].

In our laboratories, we have elected to model IAH and impaired hypoglycaemia counter-regulation in Sprague–Dawley rats or C57BL/6 mice. In both rats and mice, the McCrimmon laboratory have utilised a 4-week protocol of RH (insulin two or three times per week) administered via the IP route (0.75–1 U/kg in rats and mice and 4 U/kg in streptozotocin-induced type 1 diabetes mice; Novorapid, NovoNordisk Ltd). The Luckman laboratory have utilised the same 4-week protocol of RH with insulin administered via the SC route (1.75 U/kg; Humulin S, Eli Lilly). These protocols consistently impair plasma adrenaline release following the final hypoglycaemic episode compared with SH ([69,93] and S.M. Luckman *et al.*, unpublished).

Despite the limitations of animal modelling of IAH, important insights can be gained. Recently, using rodent models, it has been shown that IAH and impaired CRR carry hallmarks of habituation, a form of biological and cellular adaptation to a repeated stressor (in this case, hypoglycaemia) [19,69]. These insights, along with others gained from nondiabetic animal models, will need validation in diabetic models and ultimately patients with IAH.

A driver for this review was a concern that there is a lack of reproducibility of experimental findings in the field of IAH research. We postulate that much of the variation in reproducibility, both within and between research groups, is due to real differences in physiology between species. However, we must also question our own experimental design and whether improved scientific rigor can increase reproducibility.

Despite a large body of exciting mechanistic research in IAH, key questions remain unanswered (see Outstanding Questions), and an effective therapy is yet to translate to the clinic. Greater collaboration between centres with expertise in rodent and human models of IAH, as well as patients with IAH, is required to gain more translatable insights into this condition. This approach may also bring consensus in the field over which IAH models to adopt and refine. This will enable investigators to establish which findings from animal models truly translate to the human condition.

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